ENHANCED FORMATION OF RAPIDLY LABELED BILIRUBIN BY PHENOBARBITAL: HEPATIC MICROSOMAL CYTOCHROMES AS A POSSIBLE SOURCE.*

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Received June 27, 1966

In man (London et al., 1950; Gray et al., 1950), dogs (Israels et al., 1963; Schwartz et al., 1964) and rats (Robinson et al., 1965a; 1966), administration of isotopic glycine results in the appearance of labeled bile pigment within a few hours to a few days. The rapidity of this process excludes the possibility that this "early-labeled" pigment (ELP) fraction which comprises about 15 percent of all bile pigment formed (London et al., 1950; Gray et al., 1950; Robinson et al., 1965a; 1966), originates from breakdown of hemoglobin in senescent erythrocytes. It has been suggested that in part ELP may reflect turnover of heme or heme-proteins in the liver (Yamamoto et al., 1965; Schwartz and Cardinal, 1965; Robinson et al., 1965b). The possibility of direct biosynthesis of bile pigment from labeled precursors without ferroprotoporphyrin as an intermediate appears unlikely because bilirubin exhibits the 9 α configuration characteristic of fission of protoporphyrin 9a at its α-methyne bridge (Petryka et al., 1962). Moreover, in rats heme is converted rapidly and nearly quantitatively to bile pigment (Snyder and Schmid, 1965).

* Supported in part by USPHS Grant AM-07091.

Recent studies suggest that the ELP consists of multiple components; in hematologically normal rats, the early phase is entirely, and the later phase is largely derived from the liver (Robinson et al., 1965a; 1966). These conclusions are based on the observation that the magnitude and kinetics of ELP formation are not altered significantly by suppression of erythropoiesis and are reproducible with isolated perfused rat liver (Robinson et al., 1965b). The following findings are pertinent for elucidating the nature of the hepatic heme fractions that give rise to ELP:

a) The rate of glycine-2-C^{14} incorporation into bilirubin in rats is maximal between 1 and 2 hours, falls off rapidly during the next 2 hours and thereafter declines more slowly to approach base line between 2 and 3 days (Robinson et al., 1965a; 1966). This suggests that the parent heme fractions in the liver have a short biologic half-life. b) Short-term treatment with Sedormid or its congener, allylisopropylacetamide, greatly increases δ-aminolevulinic acid (ALA) synthetase (Granick, 1963; Marver et al., 1966), augments overall heme synthesis (Schmid et al., 1955; Lottsfeldt et al., 1961), leads to a moderate and late rise in microsomal cytochromes (Marver, in preparation, 1966) and inhibits catalase synthesis (Schmid et al., 1955; Price et al., 1962). Despite these effects on the liver, the rate and magnitude of ELP formation is not significantly altered (Robinson et al., 1966). c) Treatment with phenobarbital enlarges the liver and stimulates proliferation of the smooth endoplasmic reticulum. These changes are associated with enhanced NADPH-dependent drug detoxification and a great increase in microsomal Cytochromes P-450 and b_5 (Remmer and Merker, 1963; Conney et al., 1960). The pattern of phenobarbital induction of Cytochrome P-450 (Remmer and Merker, 1965) may imply that this heme protein has a rapid rate of turnover. These observations suggested that microsomal cytochromes may be a major source of ELP in the rat.

Methods

Two male Sprague-Dawley rats of 297 and 312 g received daily injec-

tions of 80 mg/kg phenobarbital for 5 consecutive days. Food was withheld for the last two days and about 18 hours before the experiments the animals were anesthetized with ether and a PE-10 polyethylene cannula* was inserted into the bile duct. The rats were placed in restraining cages for collection of bile. Hydration was maintained by continuous infusion of hypotonic saline through a catheter in a femoral vein. Two control animals of comparable weight received the same treatment except that no phenobarbital was given.

At the start of the experiments 100 μc glycine-2-C^{14} (specific activity 17 mc/m mole) was injected through the venous catheter and thereafter, bile samples were collected at 3/4, 1 1/2, 2 1/2, 4 1/2, 8, 12, 24 and 48 hours. The daily subcutaneous injection of phenobarbital and the intravenous infusion of hypotonic saline were continued throughout the experiments. The hematocrit which was monitored at frequent intervals, did not change significantly.

In all bile samples, bilirubin concentration was measured, the pigment crystallized and its specific radioactivity determined (Ostrow, et al., 1961). After the last bile collection, the rats were exsanguinated and the liver perfused with cold isotonic saline through the portal vein. An additional two male rats that received the same phenobarbital treatment but did not undergo bile duct cannulation and their controls were sacrificed 24 hours earlier.

Equal portions of liver from each pair of rats were pooled for enzyme analyses. Catalase activity was determined on 50 μl aliquots of homogenates prepared by grinding one part liver with 49 parts ice-cold distilled water in a Waring Blender, by the method of Beers and Sizer, 1952, as modified by Rechcigl et al., 1962.

The various subcellular liver fractions were obtained by differential centrifugation (Schneider, 1948). Mitochondria were separated from a 10 percent ho-

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* Clay-Adams, Inc., New York, N.Y.

mogenate in 0.25 M sucrose, while microsomes and cell sap were fractionated from a 25 percent homogenate in 0.15 M KCl containing 0.02 M sodium phosphate buffer, pH 7.0. Cytochrome P-450 was determined by measuring the CO-difference spectrum of dithionite-reduced microsome suspensions (Omura and Sato, 1964). The increment of molar extinction between 450 and 490 mμ was assumed to be 91 cm^{-1} mM^{-1} (Omura and Sato, 1963). Cytochrome b_5 was quantitated from the difference spectrum between NADH-reduced and air-saturated microsomes based on an extinction coefficient between 424 and 409 mμ of 185 cm^{-1} mM^{-1}. The mitochondrial cytochomes were determined by matrix analysis of the reduced versus oxidized difference spectra of mitochondrial suspensions (Williams, 1964). Tryptophan pyrrolase (TPO) was assayed by the method of Knox et al., 1966. One unit of tryptophan pyrrolase activity is the amount of enzyme necessary to form 1 micromole of kynurenine per hour during the linear phase of the reaction.

Results

Fractional isotope incorporation into bilirubin in phenobarbital-treated rats was increased 6-fold during the initial collection periods and approximately 3-fold during the remainder of the experiment (Table I). As shown in Figure 1, the rate of C^{14}-bilirubin formation reached its maximum between 3/4 and 1 1/2 hours, while in the controls, the apogee of the curves occurred slightly later and was much lower. These differences were reflected in an increased overall rate of bile pigment excretion in the phenobarbital-treated animals (Table 1). In normal rats, ELP comprises approximately 15 percent of all bile pigment formed (Robinson et al., 1965a; 1966). In the control animals (Table 1), this averaged 8.8 μg bilirubin per hour, compared to 54 μg after phenobarbital treatment, assuming that the bile pigment fraction derived from hemoblobin catabolism remained unchanged.

The concentration or activity of the various heme proteins in the liver

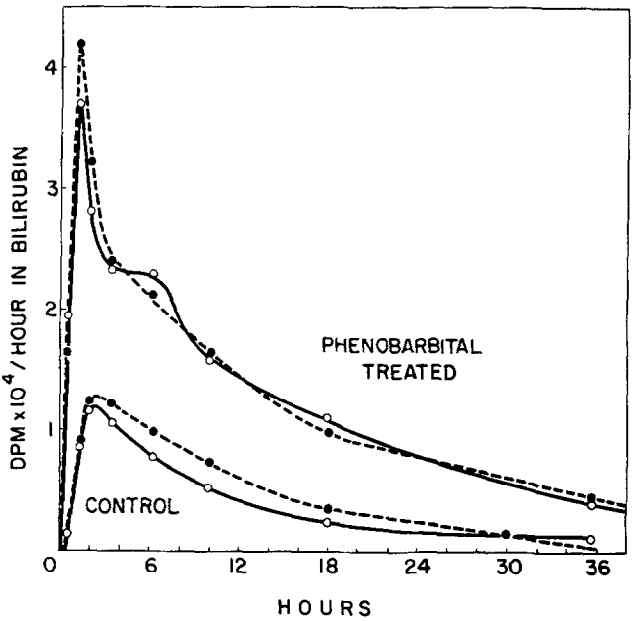

Figure 1.

Rate of C^{14}-bilirubin excretion in phenobarbital-treated and control rats, injected with 100 μc glycine-2-C^{14}.

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are listed in Table II. The only recognized heme proteins significantly increased by phenobarbital treatment were the microsomal cytochromes, and to a lesser extent, tryptophan pyrrolase. The rise in microsomal cytochromes, particularly of Cytochrome P-450, the increase in bile pigment formation from non-hemoglobin sources and the enhanced incorporation of C^{14}-glycine into bilirubin were of comparable magnitude.

Discussion

In phenobarbital-treated rats, the increase in microsomal cytochromes (Table II) remarkably parallelled the enhanced incorporation of C^{14}-glycine into bile pigment (Table I). This suggests that one or both of the cytochromes in the microsomal membranes are a major source of ELP.

Both Cytochrome P-450 and Cytochrome b_5 are present in normal liver in appreciable concentrations (Klingenberg, 1958). Induction of Cytochrome P-450

Table I

Rate of Bilirubin Excretion and Fractional C^{14}-Glycine Incorporation into Bilirubin

	Phenobarbital		Control	
Mean bilirubin excretion, μg/hr	109	95	57	60
Fractional C^{14}-glycine incorporation, %				
0 to 1 1/2 hrs	.020	.019	.003	.004
0 to 8 hrs	.090	.090	.030	.036
0 to 24 hrs	.172	.179	.053	.068
0 to 48 hrs	.218	.221	.064	.077*

* Collection terminated after 36 hours.

Table II

Hepatic Heme Enzymes in Phenobarbital-Treated Rats

Treatment	Hepatic Heme Enzymes per g Liver							
	Cytochromes, mμ moles						Catalase units	TPO units
	Microsomal		Mitochondrial					
	P-450	b_5	b	c	c_1	a		
Phenobarbital, 6 days	252	61	2.6	2.2	1.7	3.3	140	6.9
Control	30	17	3.4	2.8	2.2	4.0	143	4.9
Phenobarbital, 7 days	270	65	2.6	2.0	1.6	3.1	135	7.2
Control	28	20	3.3	2.6	2.2	4.1	129	4.8

by phenobarbital is more rapid and of greater magnitude than that of Cytochrome b_5 (Remmer and Merker, 1965). Based on a model of protein synthesis (Berlin and Schimke, 1965), the rate of induction suggests that the half-life of Cytochrome P-450 is less than 12 hours, while that of Cytochrome b_5 is considerably longer. In view of these reported differences and the present findings (Table II), it appears likely that Cytochrome P-450 is a quantitatively more important contributor than Cytochrome b_5 to ELP.

Tryptophan pyrrolase was also slightly increased after phenobarbital treatment (Table II), but its absolute concentration in the liver appears to be low. Based on a specific activity of 220 units/mg of a highly purified preparation of TPO (Schimke et al., 1965a), and an estimated molecular weight of 160,000 (Schimke et al., 1965b), 1 unit of TPO is equivalent to 0.03 mμ moles of the enzyme. On the basis of this value, the concentration of tryptophan pyrrolase in these experiments varied from 0.14 to 0.22 mμ moles per gram liver. Therefore, despite its rapid turnover (Feigelson et al., 1959; Schimke et al., 1965a), the breakdown of TPO probably contributes only slightly to bile pigment formation.

Since none of the other hepatic heme proteins was increased after treatment with phenobarbital (Table II), they probably added little to the enhanced formation of ELP. Moreover, the biologic half-life of the mitochondrial Cytochrome c is too long (Drabkin, 1951; Fletcher and Sanadi, 1961) for it to qualify as a potential source for ELP. Similarly catalase, except for a very minor fraction, has a turnover rate in excess of 24 hours (Price et al., 1962). The possibility that the enlarged ELP was derived from a greatly expanded "unassigned" heme pool in the liver cannot be completely excluded. This appears remote, however, because short treatment with allylisopropylacetamide, despite increasing ALA synthetase and overall heme synthesis in the liver, does not lead to augmented ELP formation (Robinson et al., 1966). Studies

are in progress to determine whether ELP formation resulting from rapid turnover of microsomal cytochromes involves the heme moiety only, the whole enzymes, or indeed, the endoplasmic membranes.

The findings indicate that in phenobarbital-treated animals, the amount of bilirubin formed from non-hemoglobin sources in the liver may equal that produced on sequestration of senescent erythrocytes. This observation may bear on the pathogenesis of jaundice in human liver injury, and it may require reassessment of the sites and rates of bile pigment formation.

The authors thank Mr. Stanley Larson for his excellent technical help.

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